(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 August 2001 (30.08.2001)

PCT

(10) International Publication Number
WO 01/62897 A1

(51) International Patent Classification[7]: C12N 5/06, 15/10, A61K 35/12, A01K 67/027, C12N 15/63, C07K 14/47, C12N 15/12, A61P 25/28

(21) International Application Number: PCT/EP01/02127

(22) International Filing Date: 21 February 2001 (21.02.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
00200671.6 25 February 2000 (25.02.2000) EP

(71) Applicant *(for all designated States except US)*: **VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE VZW** [BE/BE]; Rijvisschestraat 120, B-9052 Zwijnaarde (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ANNAERT, Wim** [BE/BE]; Montfortstraat 75, B-B-2550 Kontich (BE). **DE STROOPER, Bart** [BE/BE]; J. Delcordestraat 3, B-1560 Hoeilaart (BE). **HERREMAN, An** [BE/BE]; Groenstraat 35, B-2500 Lier (BE). **SCHOONJANS, Luc** [BE/BE]; Pompstraat 27, B-3012 Wilsele (BE). **SERNEELS, Lutgarde** [BE/BE]; Langendijk 21 A, B-2222 Itegem (BE).

(74) Common Representative: **VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE VZW**; Rijvisschestraat 120, B-9052 Zwijnaarde (BE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/62897 A1

(54) Title: PRESENILIN DEFICIENT MULTIPOTENT CELL LINES AND SCREENING METHODS FOR INTRAMEMBRANE REGULATED PROTEOLYTIC ACTIVITIES USING THESE LINES

(57) Abstract: The present invention relates to the field of neurological and physiological dysfunctions associated with Alzheimer's disease. More particularly to mutant embryonic stem (ES) cell lines characterized by no detectable γ-secretase activity, derived from double presenilin (PS 1 and PS 2) knock-out mice embryos. These cell lines can be used for in vitro screening of molecules and products involved in regulated intramembrane proteolysis of proteins such as the PP, the APP-like proteins, Notch, Ire-1p, and other integral membrane proteins; to identify proteases responsible for the latter proteolysis, like gamma-secretases, or proteins involved in the control of these proteolytic activities. These mutant ES cell lines can be manipulated to differentiate into fibroblast, neurons, myocytes or can be used to generate novel transgenic mice. Moreover, a reporter system comprising a chimeric molecule to detect the above mentioned intramembrane proteolysis or modulators thereof.

Presenilin deficient multipotent cell lines and screening methods for intramembrane regulated proteolytic activities using these lines

Field of the invention

The present invention relates to the field of neurological and physiological dysfunctions associated with Alzheimer's disease. Particularly, the invention relates to novel, mutant embryonic stem (ES) cell lines characterized by no detectable γ-secretase activity. More particularly the invention relates to novel, mutant embryonic stem (ES) cell lines derived from double presenilin (presenilin 1 and presenilin 2) knockout mice embryos. These novel cell lines can be used for *in vitro* screening of molecules and products involved in regulated intramembrane proteolysis of proteins such as the amyloid precursor protein, the amyloid precursor like proteins, Notch, Ire-1p, and possibly other integral membrane proteins. These cell lines can further be used to identify proteases responsible for the latter proteolysis, in particular for identifying γ-secretases, or for the identification of proteins involved in the control of these proteolytic activities. In addition, these mutant ES cell lines can be manipulated to differentiate into several specialised cell lines such as fibroblast, neurons, myocytes and other differentiated cell lines or can be used to generate novel transgenic mice. Moreover, the present invention discloses a reporter system comprising a chimeric molecule to detect the above mentioned intramembrane proteolysis or modulators thereof.

Background of the invention

Alzheimer's disease (AD) is a neurological disorder that is clinically characterized by the progressive loss of intellectual capabilities, most prominently memory, but later also by disorientation, impairment of judgement and reasoning, and ultimately full dementia. The patients finally fall into a severely debilitated, immobile state between 4 and 12 years after on-set. Worldwide, about 20 million people suffer from Alzheimer's disease. A small fraction of AD cases are caused by autosomal dominant mutations in the genes encoding presenilin (PS) proteins 1 and 2 and the amyloid-β precursor protein (APP). It has been shown that mutations in APP, PS1 and PS2 alter APP metabolism such that more of the insoluble, pathogenic Aβ peptide (Aβ42) is

produced. This Aβ42-peptide forms amyloid fibrils more readily than the Aβ40-peptide, which is normally produced via wild type APP, PS1 and PS2. These insoluble, amyloid fibrils are deposited in amyloid plaques, one of the neuropathological hallmarks in the brains of patients suffering from AD. These Aβ-peptides are generated from the amyloid precursor protein (APP), by distinct proteolytic activities. The β-secretase was recently identified and is a type I integral membrane aspartyl protease, also called BACE (Vassar *et al.*, 1999, Science 286, 735). BACE cleaves APP at the aminoterminus of the amyloid peptide sequence in APP. The elusive γ-secretase cuts APP at the carboxyterminus of the amyloid peptide. Although it is still unclear whether one, two or several different enzymes are involved in this process, it can be stated that the secretases are important and are molecular targets for drug discovery since it is believed that abnormal processing of APP is involved in the pathogenesis of both genetic and sporadic Alzheimer's Disease. It is also clear that the molecular identification of endogenous proteins involved either directly or indirectly in secretase activities is of uttermost importance.

Recent research has demonstrated the involvement of presenilins in the formation of amyloid-β through their effects on γ-secretase(s). These findings establish a genetic link between presenilins and γ-secretase(s) and make them potential molecular targets for developing compounds to prevent or treat AD.

Presenilins (PS) are polytransmembrane proteins located in the endoplasmic reticulum and the early Golgi apparatus. Missense mutations cause familiar Alzheimer's disease (AD) in a dominant fashion. The exact pathogenic mechanism underlying the disease process is not fully unravelled, but it is fairly established that most PS missence mutations effect the processing of the amyloid precursor protein (APP), resulting in an increased generation of the longer form of the amyloid peptide (the Aβ42-peptide) which is a major component of the amyloid plaques in patients as stated above.

The inactivation of the PS1-gene in mice results in a severe lethal phenotype, characterised by late embryonic lethality, disturbed somitogenesis, mid-line closure deficiencies and malformations of the central nervous system, most significantly underdevelopment of the subventricular zone and a neuronal migration disorder mimicking human lissencephaly type II (Hartmann *et al.*, 1999, Curr. Biol.9, 719). Cell biological studies in PS1-deficient neurons have demonstrated that PS1 deficiency interferes with the γ-secretase-mediated proteolysis of the transmembrane domain of APP and an estimated reduction of 85% γ-secretase-mediated proteolysis was

observed (De Strooper *et al.*, 1998, Nature 391, 387). However, it is unknown which additional factors modulate the activity of the γ-secretase.

Presenilin 1 appears also to be involved in the proteolytic processing of the transmembrane domain of other proteins like Notch, a signaling protein involved in cell fate decisions (De Strooper *et al.*, 1999, Nature 398, 518), and possibly Ire1p, a protein involved in the control of the unfolded protein response (Niwa *et al.*, 1999, Cell 99, 691). This type of proteolytic processing has been recently called "regulated intramembrane proteolysis" (rip) (Brown et al. (2000) Cell 100, 391). Recently, Wolfe and colleagues (Wolfe *et al.*, 1999, Nature 398, 513) proposed that PS1 itself is an unusual aspartyl protease. Owing to their involvement in the cleavage of APP, the presenilins may turn out to be the long-sought-after γ-secretase. Aspartyl proteases, like the presenilins, require two aligned aspartate residues in their catalytic domain. It should be emphasized, however, that direct evidence for the conclusion that PS1 or PS2 represent the γ-secretase activity itself is still lacking and that direct evidence that presenilins have catalytic activity has not been provided. Moreover it is unclear whether all proteolytic cleavages in which presenilin is involved can be performed by one identical protease, since the primary amino acid sequences of the different cleaved proteins is quite variable. It is therefore certainly possible that the presenilins influence γ-secretase activity indirectly. Presenilins may for instance control membrane insertion of γ-secretase or may behave as co-factors stimulating their catalytic activity. An analogy can be drawn with the regulation of the site 1 cleavage of sterol regulatory element binding proteins, which is controlled by a multitransmembrane cleavage-activating protein located in the ER.

Recent evidence also implies the presenilins in Wnt/β-catenin signaling (Zhang et al., Nature 395:698-702, 1998; Kang et al., J. Neurosci.19: 4229-4237, 1999). While their exact role in this pathway remains controversial, it is established that presenilins can interact with β-catenin.

A major problem with the hypothesis that presenilins are proteases is their subcellular localization. Presenilin proteins have been localized to early transport compartments, whereas abundant γ-secretase activity is thought to be associated with the late transport compartments and the endosomal pathway (Annaert *et al.*, 1999, J. of Cell Biology 147, 277).

The interpretation of all studies performed until now have been complicated by the fact that at least two presenilin genes do exist. Most studies did not take into account the

influence of the PS2-gene. In contrast to PS1 knock-out mice, PS2 knock-out mice are viable and fertile and develop only mild pulmonary fibrosis and hemorrhage with age (Herreman *et al.*, 1999, Proc.Natl.Acad.Sciences 96, 11872). Quite surprisingly and unexpectedly, the absence of PS2 does not detectably alter the processing of amyloid precursor protein.

In a further step the complete deletion of both PS2 and PS1 genes was therefore pursued. The phenotype of these mice closely resembles the phenotype of mice that are fully deficient in Notch-1. These observations demonstrate that PS1 and PS2 have partially overlapping functions and that PS1 is essential and PS2 is redundant for normal Notch signalling during mammalian embryological development. Biochemical analysis of the exact effects of the double PS deletion on Notch signaling, APP processing, the UPR and other biological processes, is however hampered by the fact that only a limited number of cells can be obtained from such embryos. This has been circumvented by immortalizing these cells using transfection with large T or myc cDNA constructs. Although this allows to obtain large amounts of presenilin negative cells, these procedures also largely interfere with important cellular signaling mechanisms and also lead to genetic instability of the cells. It is therefore difficult to assess correctly to what extent phenotypical alterations in these cells are caused by the presenilin deficiency in se or by secondary alterations caused by the immortalisation procedure. It is thus clear that the development of clean and genetically stable cell lines, without activity of PS1 and PS2, is needed in order to understand the above described biological pathways, and especially the role of the presenilins, their mutations and deficiencies, in the pathogenesis of AD.

The present invention provides embryonic stem (ES) cell lines generated from double presenilin (PS1 and PS2) knockout mice. Surprisingly, given the residual γ-secretase activity in PS1 knock out cells and the absence of effects on γ-secretase activity in PS2 knock out cells, it was found that γ-secretase activity dropped to an undetectable level in these mutant cell lines. Accordingly, the latter cell lines can be used to screen for γ-secretase activity and modulators thereof. The present invention also provides a reporter system to detect γ-secretase activity and modulators thereof.

Figure Legends

Figure 1:

Southern blot analysis of ES clones obtained from individual blastocysts.

ES cells were harvested and genomic DNA was isolated. After restriction with KpnI, Southern blotting was performed using the 5' PS1 probe (Saftig *et al.*, Molecular biology of Alzheimer's Disease, Harwood Academic publishers, editor Christian Haass, pp 235-246). A 6.8 kb and 4.5 kb band is obtained indicating respectively a wild type or a targeted PS1 allele. As one can observe lanes a, b, f, g and i are double presenilin mutant ES cells.

Figure 2:

Lower βA4 production in double PS1 and PS2 knock out ES cells

ES cells were transduced with recombinant Semliki Forest Virus to induce expression of APP/Sw (De Strooper *et al.*, 1995, EMBO J. 14, 4932)). After transduction, cells were metabolically labeled with ^{35}S-methionine (500 µCi/ml) and after 4 hours the conditioned medium was collected and cells were lysed. The conditioned medium was used in an immunopreciptiation reaction using amyloid peptide specific antibodies B7/7 (De Strooper *et al.*, 1998, Nature 391, 387). No amyloid peptide production is observed in the double deficient ES cells.

Figure 3:

Accumulation of APP carboxyterminal fragments

ES cells were transduced (APPSw) or not transduced (Co) with recombinant Semliki Forest Virus to induce expression of APP/Sw. Cells were labeled as in panel B and cell extracts were generated as described (De Strooper *et al.*, 1995, EMBO J. 14, 4932). APP antibodies B11/4 recognizing the carboxyterminal end of APP were used to immunoprecipitate APP (holo-APP) and carboxyterminal fragments of APP (α-stubs and β-stubs). An accumulation of APP carboxyterminal fragments is observed in the first lane, indicating inhibition of the normal turn over of these fragments in the double knock out cells (De Strooper *et al.*, 1998, Nature 391, 387).

Figure 4:

A cell based γ-secretase assay

Panel A: The APP ectodomain and transmembrane domain are fused to the intracellular domain of Notch. Luciferase cDNA is fused to the Hes-1 promotor fragment as indicated in the text. Cells are transfected with these constructs. Proteolytic release of the Notch intracellular domain by a γ-secretase activity induces luciferase activity as indicated, allowing to monitor efficiently γ-secretase activity. (CSL is present in the transfected cells).

Panel B: Different constructs are displayed. The first series are chimeric proteins containing progressively shortened Notch intracellular domain fragments fused to wild type APP (see text for details). The next constuct contains the Swedish mutation of APP to increase β-secretase cleavage. The last construct is similar to the first construct, but the APP ectodomain was truncated at the β-secretase site.

Panel C: pSG5APP-NIC and pSG5APPsw-NIC constructs were transfected in Cos and Hela cells. Reaction with antibodies against the N terminus of APP (22C11), or against the cytoplasmic domain of APP or with the monoclonal 9E10 (myc tag) demonstrated in western blotting a protein with a molecular mass around 150 kDa corresponding to the predicted fusion protein (indicated as APPNIC). α or β secretase cleaves the extracellular domain of APP producing a soluble ectodomain APPs and a membrane associated carboxy-terminal fragments (APP/NIC CT fragments). Amyloid-β peptide and p3 fragment are also produced, as indicated in the final panel. The production of amyloid and p3 peptide is less efficient from the chimeric construct than from wild type APP. One possible reason is that the endocytosis signals in the APP cytoplasmic tail, important for amyloid production in wild type APP are not present in the chimeric protein.

Panel D: Results with different constructs are displayed. Significant induction of luciferase activity is shown after transfection with the chimeric constructs. The different APP/NIC chimeric proteins have very similar induction efficiencies.

Aims and detailed description of the present invention

The present invention aims at providing embryonic stem cell lines in which the residual γ-secretase activity is reduced by more than 90%, preferentially more than 99%, and

more preferentially more than 99.9%, compared to the γ-secretase activity in embryonic stem cell lines derived from corresponding wild-type mice.

A particular ES cell line of the present invention has been deposited with the Belgian Coordinated collections of Microorganisms (BCCMTM), Laboratorium voor Moleculaire Biologie – Plasmidencollectie (LMBP), Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium, and has been given accession number: LMBP 5472CB.

The present invention thus provides a clean mutant mammalian environment which allows to exclude the contribution of endogenously expressed presenilins to any induced γ-secretase activity or other proteolytic activities. Furthermore, such cell lines provide a perfect background to investigate the biochemical effects of transfected presenilins containing mutations, since no interference from endogenously wild type presenilins is possible.

In a preferred embodiment, the double presenilin mutant ES cell line can be used as a tool to isolate and identify γ-secretase candidates and genetic modulators of γ-secretase activity. By isolation it is meant that standard molecular biology tools such as complementation, screening or selection cloning methods with a genomic or cDNA library are used to transfect the cells and to induce γ-secretase activity. It is clear that also recombinant virus libraries, such as adenoviral, lentiviral or retroviral libraries can be used.

In another embodiment, the double presenilin mutant ES cell line can be used to generate membranes or protein extracts that can be complemented biochemically with fragments of presenilin or other proteins to reconstitute gamma-secretase activity *in vitro* (Li et al, Proc. Natl. Acad. Sci 97; 6138-43, 2000). The aforementioned methodology are only examples and do not rule out other possible approaches of using these cells that could lead to potential γ-secretase candidates. Since presenilins are required for the maintenance of γ-secretase activity in normal conditions, it is anticipated that screening assays will yield parts of γ-secretase that are devoid of putative regulatory domains that interact with presenilins. Such partial clones can then be used to obtain the complete cDNA of γ-secretase and /or other proteases involved in regulated intramembrane proteolysis (see above), more in particular of APP. Restoration of proteolytic activity can be followed by different means, to give only a few examples: ELISA assays or other assays measuring amyloid peptide production, or assays measuring Notch cleavage using luciferase reporter systems or other. To

increase the sensitivity of such assays it can be considered to stably transfect the ES cells with cDNA's encoding APP (human, containing clinical or synthetic mutations), Notch (possibly mutated or modified), or other proteins and reporters useful for such assays.

In another embodiment of the invention the double mutant presenilin ES cell line can be used as a cellular background to express presenilin clinical mutations. Such a cell line can then be used to screen for inhibitors that specifically inhibit the production of pathogenic amyloid-β42-peptide. Indeed, the double mutant presenilin ES cell line transformed with a Alzheimer's disease causing PS1 mutation is predicted to produce predominantly the amyloid-β42 form whereas the mutant ES cell line transformed with the wild type PS1 is predicted to produce mostly the non-pathogenic amyloid-β40 peptide. These cell lines can thus be used in differential drug screening approaches to identify compounds which inhibit preferentially the amyloid-β42 formation and not the amyloid-β40 peptide generation. By comparing the differential effect of compounds on the amyloid peptide production in the mutant cell lines overexpressing wild type presenilin and clinical mutant presenilin, compounds affecting the pathological amyloid peptide (amyloid-β42 peptide) production can specifically be detected. A compound able to interfere with the formation of amyloid-β42 peptide and not with the formation of amyloid-β40 peptide should at least have a 20% reduced amyloid-β42 peptide formation, preferentially at least a 50% reduced amyloid-β42 peptide formation and more preferentially at least a 90% reduced amyloid-β42 peptide formation.

In another embodiment the invention provides a method for the production of a pharmaceutical composition comprising the usage of an embryonic stem cell line to identify a gene coding for a protein having gamma-secretase activity or a compound that specifically interferes with the formation of the Aβ42 – peptide and not with the formation of the Aβ40 – peptide, and further more mixing the gene or compound identified or a derivative or homologue thereof with a pharmaceutically acceptable carrier. The administration of a gene or compound or a pharmaceutically acceptable salt thereof may be by way of oral, inhaled or parenteral administration. The active compound may be administered alone or preferably formulated as a pharmaceutical composition. A unit dose will normally contain 0.01 to 50 mg for example 0.01 to 10 mg, or 0.05 to 2 mg of compound or a pharmaceutically acceptable salt thereof. Unit doses will normally be administered once or more than once a day, for example 2,

3, or 4 times a day, more usually 1 to 3 times a day, such that the total daily dose is normally in the range of 0.0001 to 1 mg/kg; thus a suitable total daily dose for a 70 kg adult is 0.01 to 50 mg, for example 0.01 to 10 mg or more usually 0.05 to 10 mg. It is greatly preferred that the compound or a pharmaceutically acceptable salt thereof is administered in the form of a unit-dose composition, such as a unit dose oral, parenteral, or inhaled composition. Such compositions are prepared by admixture and are suitably adapted for oral, inhaled or parenteral administration, and as such may be in the form of tablets, capsules, oral liquid preparations, powders, granules, lozenges, reconstitutable powders, injectable and infusable solutions or suspensions or suppositories or aerosols. Tablets and capsules for oral administration are usually presented in a unit dose, and contain conventional excipients such as binding agents, fillers, diluents, tabletting agents, lubricants, disintegrants, colourants, flavourings, and wetting agents. The tablets may be coated according to well-known methods in the art. Suitable fillers for use include cellulose, mannitol, lactose and other similar agents. Suitable disintegrants include starch, polyvinylpyrrolidone and starch derivatives such as sodium starch glycollate. Suitable lubricants include, for example, magnesium stearate. Suitable pharmaceutically acceptable wetting agents include sodium lauryl sulphate. These solid oral compositions may be prepared by conventional methods of blending, filling, tabletting or the like. Repeated blending operations may be used to distribute the active agent throughout those compositions employing large quantities of fillers. Such operations are, of course, conventional in the art. Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups, or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, for example sorbitol, syrup, methyl cellulose, gelatin, hydroxyethylcellulose, carboxymethyl cellulose, aluminium stearate gel or hydrogenated edible fats, emulsifying agents, for example lecithin, sorbitan monooleate, or acacia; non-aqueous vehicles (which may include edible oils), for example, almond oil, fractionated coconut oil, oily esters such as esters of glycerine, propylene glycol, or ethyl alcohol; preservatives, for example methyl or propyl p-hydroxybenzoate or sorbic acid, and if desired conventional flavouring or colouring agents. Oral formulations also include conventional sustained release formulations, such as tablets or granules having an enteric coating. Preferably, compositions for inhalation are presented for administration to the respiratory tract as a snuff or an

aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose. In such a case the particles of active compound suitably have diameters of less than 50 microns, preferably less than 10 microns, for example between 1 and 5 microns, such as between 2 and 5 microns. A favored inhaled dose will be in the range of 0.05 to 2 mg, for example 0.05 to 0.5 mg, 0.1 to 1 mg or 0.5 to 2 mg. For parenteral administration, fluid unit dose forms are prepared containing a compound of the present invention and a sterile vehicle. The active compound, depending on the vehicle and the concentration, can be either suspended or dissolved. Parenteral solutions are normally prepared by dissolving the compound in a vehicle and filter sterilising before filling into a suitable vial or ampoule and sealing. Advantageously, adjuvants such as a local anaesthetic, preservatives and buffering agents are also dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under vacuum. Parenteral suspensions are prepared in substantially the same manner except that the compound is suspended in the vehicle instead of being dissolved and sterilised by exposure to ethylene oxide before suspending in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of the active compound. Where appropriate, small amounts of bronchodilators for example sympathomimetic amines such as isoprenaline, isoetharine, salbutamol, phenylephrine and ephedrine; xanthine derivatives such as theophylline and aminophylline and corticosteroids such as prednisolone and adrenal stimulants such as ACTH may be included. As is common practice, the compositions will usually be accompanied by written or printed directions for use in the medical treatment concerned.

Another embodiment involves the differentiation of the obtained ES cell lines towards neurons and in particular towards post-mitotic neurons.

In another embodiment the double mutant presenilin ES cell line can be used to differentiate into many cell lineages, including heart muscle cells, blood islands, pigmented cells, macrophages, epithelia, and fat-producing adipocytes.

In another embodiment the double mutant presenilin ES cell line can be used to clarify the role presenilins play in the unfolded protein response (UPR-response).

In another embodiment the double mutant presenilin ES cell line can be transformed with a specific presenilin mutant being a pathogenic presenilin Alzheimer's disease causing gene. The resulting transformed mutant ES cell line with the specific presenilin

mutant can be injected back into a recipient blastocyst that is then carried to term in a female host. In this way it is possible to generate very specific, clean transgenic mice in which there is no interference of the wild type presenilin homologous gene neither of the other presenilin genes.

In another embodiment the double mutant presenilin ES cell line can be used to unravel the role of Notch in differentiation or to unravel the Notch signalling pathway. As a preferred but not limited example the double mutant presenilin ES cell line can be transformed with an inducible PS1 gene and in a gene expression profiling experiment the gene expression can be monitored before and after induced expression of PS1.

In another embodiment the double mutant presenilin ES cell line can be used to unravel the presumed role PS2 is playing in inducing apoptosis.

Another embodiment is the use of these mutant ES cell lines in a cell free assay whereby vesicle budding from ER membranes is studied. The profiling of the protein composition of the generated transport vesicles can then be investigated by two dimensional electrophoresis and amino acid sequencing of protein spots that are either increased or decreased in comparison with spots present in the profile of similar gel profiles obtained from wild type cells.

Another aspect of the invention relates to the realisation, especially in the field of cell signalling, that certain transmembrane proteins can be cleaved within the transmembrane domain to liberate cytosolic fragments that enter the nucleus to control gene transcription. This mechanism, called regulated intramembrane proteolysis (Rip), influences processes as diverse as cellular differentiation, lipid metabolism, and the response to unfolded proteins (Brown et al. (2000) Cell 100, 391). Currently five animal proteins are known or postulated to undergo Rip: SREBP, APP, Notch, Ire1 and ATF6 (Brown et al. (2000) Cell 100, 391). In some of these examples, the information is still fragmentary, and in no case do we have a complete picture of the processing events. Furthermore there are no reporter systems described that can detect Rip and also the processing enzymes (e.g. proteases) have not been identified with certainty. The present invention provides a system to detect Rip and to identify processing enzymes (e.g. proteases) and modulators of said processing enzymes.

Thus, a reporter system was generated that can be used to detect intramembrane proteolytic processing. Said reporter system comprises a chimeric molecule further comprising a fusion between a transcription factor and a transmembrane domain that is known to be a substrate for proteolytic processing, and a reporter construct that can

detect said proteolytic processing of said chimeric transcription factor. When intramembrane cleavage occurs said chimeric transcription factor is not kept sequestered anymore in the membrane and can shuttle to the nucleus where it activates the expression of a reporter gene. Said reporter gene is kept under control of an inducible promoter of which activity is dependent on the release of the specific, membrane sequestered transcription factor.

In a further embodiment the chimeric molecule that is used in the reporter system comprises a fusion between the intracellular domain of Notch and the transmembrane domain of APP. Said reporter system can be used to detect intramembrane proteolytic processing by γ-secretase. The transmembrane domain of APP comprises said γ-secretase cleavage site.

In yet another embodiment the chimeric molecule of the reporter system comprises a fusion between the intracellular domain of Notch and the ecto- and transmembrane domains of APP and is set forth by SEQ ID NO: 13. In SEQ ID NO: 13 APP starts at nucleotide 275 and ends at 2266. The transmembrane domain of APP starts at 2147 and ends at 2208. The intracellular domain of Notch starts at 2273 and ends at 4249. The myc tag starts at 4256 and ends at 4285. The stop codon of the chimeric protein is between 4286-4288.

In yet another embodiment the reporter system of the present invention can be used to screen for modulators and/or proteases for intramembrane proteolytic processing. In a particular embodiment the genetic background that is used to screen for said modulators and/or proteases is devoid of the activity of intramembrane proteolytic processing. In such a genetic background there is no background activation of the reporter construct since the chimeric transcription factor is maintained in the membrane. For example, the double presenilin ES cell line of the present invention is a perfect tool for the isolation of the γ-secretase or modulators of γ-secretase activity. By 'isolation' it is preferentially meant 'screening', and more preferentially 'selection'. As has been said before γ-secretase is an enzyme involved in Rip of the transmembrane domain of APP. In order to establish a successful cloning experiment the mutant ES cell line is first adapted into a suitable reporter ES cell line with the introduction, by transfection, of the above described reporter system. Screening for γ-secretase (a protease) or modulators of γ-secretase activity can be carried out with a suitable reporter gene, such as the green fluorescent protein. However, one can also select for γ-secretase or modulators of γ-secretase activity by use of a selection marker, such as

the neomycin phosphotransferase gene, under control of an inducible promoter which activity is dependent on the release of the specific, sequestered transcription factor. As such, the transcription factor is released from its membrane localisation and, consequently, provides antibiotic resistance after cleavage by the γ-secretase or γ-secretase modulators. Selection or screening for the γ-secretase activity is carried out by the transfection of a genetic library to the reporter ES cell line. Preferentially this library is a mammalian genomic library and more preferentially the library is a cDNA library under control of a suitable promoter and even more preferentially the cDNA library is of neuronal origin. It is clear that also recombinant virus libraries, such as adenoviral, lentiviral or retroviral libraries can be used. The aforementioned libraries are only examples and do not rule out other possible cloning approaches that could lead to the identification of potential γ-secretase candidates or genetic modulators of γ-secretase. By modulators, here it is meant compounds or genes that influence the activity of the proteases sought, as such a modulator can enhance (activator) or diminish (suppressor) the activity. For instance, but not limited, a modulator of γ-secretase can be a protein binding to γ-secretase or a protein that forms part of a multi-protein complex that has γ-secretase activity.

In another embodiment the reporter system of the present invention is used to screen for modulators and/or proteases in a cell line with a wild type genetic background, wherein the protease(s) that provoke the intramembrane proteolytic processing are present. In such a cell line there is usually a constitutive expression level of the reporter construct. As such, a selection or screening system can be set up by comparing the expression level of the reporter construct in said cell line with the same but transfected or compound treated cell line. As such, in a screening or selection experiment, a higher level of the protease, such as for example the γ-secretase or a modulator thereof, in said cell line (e.g. by transfection with at least one gene), can be detected by a higher activation of the reporter construct with respect to the non-transfected cell line. Conversely, a lower activity of the γ-secretase can also be detected by a specific compound or a modulator when compared with the non-transfected or non compound treated cell line. Finally, in another embodiment a process is described to construct a reporter system. Said process comprises the formation of two specific constructs. The first construct is made by splicing the sequence of a transcription factor to a transmembrane domain that is known to be a

substrate for proteolytic processing and the second construct is made by splicing of a genetic element that is responsive to said transcription factor to a reporter gene.

The following paragraphs clarify some terms used above and in the claims:

The 'ES cell' is preferably a culture cell established from the inner cell mass of a murine embryo, usually isolated at the age of 3.5 days. 'Compound' means any chemical or biological compound, including simple or complex inorganic or organic molecules, peptides, peptido-mimetics, proteins, antibodies, carbohydrates, nucleic acids or derivatives thereof. 'Wild type' is an animal (e.g. a mouse) or cell line that is isogenic with the mutant animal (e.g. mouse) or cell line except for the mutation or mutations induced in said mutant. 'Gene' means a functional promoter sequence fused to a sequence that can be transcribed, due to the activity of said promoter, into mRNA, and subsequently translated into protein, eventually after processing the mRNA by a process such as mRNA splicing. Said promoter may be the endogenous promoter of the transcribed sequence, or a heterologous promoter. 'Mutant (ES) cell line' is a (ES) cell line genetically modified by a procedure known to the people skilled in the art such as random mutagenesis, retroviral or adenoviral or lentiviral insertion, transposon mutagenesis, heterologous or homologous recombination. 'A mutant gene' is a variant of the wild type gene that differs from the wild type gene by a change, insertion and/or deletion of at least one amino acid. 'Transgenic mouse' is a mouse derived from the mutant ES cell line. It also includes an individual animal in all stages of development, including embryonic and fetal stages. A 'transgenic animal' is an animal containing one or more cells bearing genetic information received, directly or indirectly, by deliberate genetic manipulation at a subcellular level, such as by microinjection or infection with recombinant virus. This introduced DNA molecule may be integrated within a chromosome, or it may be extra-chromosomally replicating DNA. The term 'germ cell-line transgenic animal' refers to a transgenic animal in which the genetic information was introduced into a germ line cell, thereby conferring the ability to transfer the information to offspring. If such offspring in fact possess some or all of that information, then they too are transgenic animals. The information may be foreign to the species of animal to which the recipient belongs, foreign only to the particular individual recipient, or genetic information already possessed by the recipient. In the last case, the introduced gene may be differently expressed compared to the native endogenous

gene. A variant transgenic animal is a knockout animal possibly prepared according to Capecchi, 1989, Trends Genet. 5, 70. A 'reporter gene' is generally incorporated in a 'reporter construct'. A 'reporter gene' is a DNA molecule that expresses a detectable gene product, which may be RNA or protein. The detection may be accomplished by any method known to one skilled in the art. For example, detection of mRNA expression may be accomplished by using Northern blots and detection of protein may be accomplished by staining with antibodies specific to the protein. Preferred reporter genes are those that are readily detectable comprising chloramphenicol acetyl transferase, luciferase, beta-galactosidase and alkaline phosphatase. Other reporter genes are genes providing antibiotic resistance such as the neomycine phosphotransferase gene. As used herein, "APP695" refers to the 695 amino acid residue long polypeptide encoded by the human APP gene (Kitaguchi et al. (1988) Nature 331:530). A 'chimeric molecule' means a molecule not naturally encountered in nature, in this invention the chimeric molecule is obtained by 'splicing' part of the genetic information residing from two different genes encoding proteins. 'splicing' is a term used by molecular biologists and here means 'joined' by known methods in genetic engineering (e.g. PCR-cloning, restriction enzyme mediated cloning). A 'transmembrane domain' is a protein domain that crosses a membrane (e.g. endoplasmic reticulum, plasma membrane). The terms 'transformed' or 'transfected' are used interchangeably and refer to the process by which heterologous DNA or RNA is transferred or introduced into an appropriate host cell.

Examples

I. Results

1. Generation of double presenilin knockout mice

The generation of PS1+/- and PS2-/- mice has been described previously (De Strooper *et al.*; 1998, Nature 391, 387, Herreman *et al.*1999, Proc.Natl.Acad.Sciences 96, 11872). All mice used have a C57B6/J black x 129Sv genetic background.

PS2-/- mice were crossed with PS1+/- mice to obtain PS1+/-PS2+/- double heterozygous mice. Double heterozygotes were then crossed with PS2-/- homozygous mice to obtain PS1+/-PS2-/- mice. Even with only one active PS1 gene left, these mice remain completely viable and fertile. Liveborn double homozygous offspring from these

heterozygous intercrosses could not be detected. At E9.5 homozygous PS1-/-PS2-/- embryos could be recovered in a nearly Mendelian distribution (15/66), but embryos were developmentally retarded by approximately halve a day when compared to heterozygous littermates. Vasculogenesis of the yolk sac was delayed in most of the mutants. Although an initial vascular plexus and primitive red blood cells had formed, organisation into a discrete network of vitelline vessels was always lacking. Furthermore, yolk sacs did not expand properly and often had a blistered appearance. The embryo itself was always devoid of blood circulation and appeared posteriorly truncated. Heart development was largely unaffected, with the exception of an occasional enlarged pericardial sac. Somitogenesis had begun and turning occured in the majority of the mutants. The optic and otic vesicle, the first branchial arch and the forelimb buds were visible. Mutants had a vestigial fore- and hindbrain, and fusion of headfolds was delayed. The neural tube had often a kinked appearance, which may be secondary to the circulation problems. This phenotype of the double deficient embryos is clearly different from that of PS2-/- embryos which appear normal and PS1-/- embryos which are only marginally retarded at E9.

2. Generation of double presenilin deficient ES cells

It is well known in the art that cells from the inner cell mass of mammalian blastocysts can be maintained in tissue culture under conditions where they can be propagated indefinitely as pluripotent embryonic stem (ES) cells (Thomson *et al.*, 1998, Science 282, 1145). As such blastocysts from double presenilin knockout mice were rescued from 2 days old embryos and the mutant embryonic stem cells were generated by cultivation *in vitro* according to the method of US 6103523. In Figure 1 a Southern blot is shown demonstrating the presence of wild type or knockout alleles of presenilin 1 in ES cell lines obtained from blastocysts generated by mating $PS^{+/-}PS2^{-/-}$ mice.

3. Determination of γ-secretase activity

In figures 2 and 3 the analysis of APP processing, containing the Swedish clinical mutation, in a double presenilin knockout background is presented. It is shown that no amyloid peptide production is observed in the double presenilin deficient ES cells.

The total gamma-secretase activity in cells is measured by assessing the release of the amyloid peptide in the culture medium from cells transfected with cDNA coding for either wt APP or APP containing the Swedisch type of mutation. Measurement is done

by ELISA, mass spectometry, western blot or double immune precipitation in combination with phosphor imaging or by any other means that allow to determine the amount of amyloid peptide secreted into the medium. The secretion of amyloid peptide in cells expressing wild type PS1 is taken as reference. Increases or decreases in amyloid peptide secretion by the test cells are expressed as the fraction of the reference.

4. Differentiation of double presenilin deficient ES cells

Stem cells have both the capacity to self-renew, that is, to divide and create additional stem cells, and also to differentiate along a specified molecular pathway. Embryonic stem cells are very nearly totipotent, reserving the elite privileges of choosing among most if not all of the differentiation pathways that specify the animal. Upon LIF withdrawal, ES cells cultured on non-adhesive tissue culture surface spontaneously aggregate into embryo-like bodies, where they differentiate and spawn many cell lineages, including beating heart muscle cells, blood islands, neurons, pigmented cells, macrophages, epithelia, and fat-producing adipocytes

In one speficic, but not limited, example aggregates of these mutant, cultured mouse ES cells can be differentiated into neuronal precursor cells and functional postmitotic neurons (Okabe *et al.*, 1996, Mech. Dev. 59, 89). This is achieved by taking aggregates of cultured mutant ES cells and propagating them in medium supplemented with insulin, transferrin, seleniumchloride and fibronectin to select for CNS stem cells. These CNS stem cells are proliferated in the presence of mitogen, bFGF. Further differentiation of the stem cells into mature neurons is achieved by withdrawal of bFGF.

This experimental system provides a powerful tool for analyzing the molecular mechanisms controlling the functions of these neurons *in vitro*.

In another example these mutant ES cells can be differentiated into adipocytes. This is achieved by culturing the embryoid bodies in medium containing retinoic acid and subsequently plating them in medium supplemented with insulin and triiodothyronine.

This system provides a model for the further characterization of the role of genes, expressed during the adipocyte development program, like Notch-1, which is required for adipogenesis.

5. Development of a reporter system for gamma-secretase and use of said system in cell lines.

The principle of the assay is depicted in figure 4A. An APP/Notch chimeric protein is generated. This protein contains the APP ecto- and transmembrane domains, fused to the Notch intracellular domain. The Notch intracellular domain (NIC), when cleaved, translocates to the nucleus and activates a reporter gene construct containing a defined part of the Hes 1 promotor controlling Luciferase expression. Thus proteolytic cleavage of the chimeric protein is directly linked to luciferase activity. The chimeric protein and the luciferase reporter are transiently or stably transfected in Hela-cells, HEK293 cells, COS-cells, Embryonic stem cells and other. The chimeric protein is cleaved by α-secretase, β-secretase and γ-secretase. The γ-secretase cleavage of the construct is dependent on PS expression. In ES cells lacking PS1 and PS2 transfected with the luciferase reporter and the chimeric protein and a control plasmid, no significant Luciferase activity is induced. If an expression plasmid coding for PS1 is cotransfected however, luciferase activity is induced. Transfection experiments using the intracellular domain of the chimeric protein alone (bypassing the need for γ-cleavage) results in much stronger activity of the luciferase reporter in Hela cells than obtained with the APP/Notch chimeric. Therefore both decreased and increased γ-secretase cleavage can be assessed with this assay.

This assay can be used as a screening assay for compounds that inhibit or stimulate γ-secretase activity. In such assays compounds are added to cells expressing the chimeric and reporter protein. After a defined period of time (24 or 48 hours, but shorter periods of time can be chosen), luciferase activity is measured in the treated cells and in the control cells. Changes in luciferase activity are an indicator of decreased or increased γ-secretase activity. Compounds that selectively decrease luciferase activity in cells expressing the chimeric protein and reporter but not in cells transfected with the intracellular part of the chimeric protein alone are not toxic to the cells and are likely specific inhibitors for the γ-secretase.

This assay can also be used to screen for cDNA's coding for proteins that modulate γ-secretase activity. In this type of experiments cDNA's from a cDNA library either using classical transfection protocols or using viral transduction (adeno-, Semliki Forest- or other viral vectors) are transfected into cells or cell lines expressing transiently or stable the chimeric protein and the reporter. Positive hits (significant up- or down regulation of luciferase activity) can be selected from these screens. The

plasmids coding for individual cDNA's or for pools of cDNA's can be used for further screens in the same assay or for experiments in neurons or cell lines to confirm their effects in APP processing. Pools of plasmids can be further subdivided and tested in consecutive rounds of transfection and subdivision until plasmids encoding a single type of cDNA are obtained. This cDNA is anticipated to encode γ-secretase, an active γ-secretase fragment or modulators that either activate or inhibit this enzyme. Further validation of positive clones can be performed by transfecting or transducing the obtained cDNA's into neuronal cells or in cell lines, and to analyze APP processing. These cells can be transfected with APP constructs encoding human APP.

A third application of the assay is the screening for ligands of APP. This is based on the observation that binding of ligands to Notch induces proteolytic processing of Notch and cell signaling. It is expected that in analogy, APP ligands binding to the ectodomain of the chimeric protein will induce γ-secretase processing, which consecutively will cause enhanced luciferase activity. Serum and plasma, cerebrospinal fluid, or other body fluids, cell extracts, conditioned medium of cells in culture, membrane enriched extracts of cells in culture, membrane enriched fractions of tissues, in particular brain, or fractions thereof can be used as starting material and added to (stably) transfected chimeric protein expressing cells. If these materials contain APP ligands it is expected that they will result in increased luciferase activity. Further purification of these ligands can be obtained by classical fractionation (gel filtration, anion exhange or other chromatographic procedures, ultracentrifugation or specific precipitation using high salt concentrations and other methods). Purified material can be sequenced using Edman degradation or Maldi-Toff. It is also possible to use cells transfected with cDNA's or pools of cDNA's and to add them to the cells transfected with the chimeric construct and the reporter and to screen for enhanced luciferase activity. If a membrane bound ligand for APP is expressed in these cells, it is predicted that γ-secretase activity and consequently luciferase activity will be increased. By subdividing the plasmid pools and transfecting these subpools in the cells, and by screening these subpools for their effect in the assay, it is possible to obtain progressively enriched pools of plasmids encoding cDNA's for potential ligands of APP. By repeating this type of experiment until one single cDNA clone is obtained, it is possible to identify APP ligands.

6. Development of a cell free assay

The mutant ES cell line can be used as the basis for a cell free assay following the generation and release of transport vesicles from endoplasmic reticulum. The same type of studies has been critical for the unravelling of the molecular sorting machinery in eukaryotic cells. One can isolate ER-fractions from wild type and PS1-/-mice and the export budding from the ER can be reconstituted using small GTP binding proteins and recombinant COPII coat proteins. Newly formed vesicles can be isolated and analyzed for amyloid peptide generation. The molecular composition of the vesicles can be analyzed using 2D gel electrophoresis combined with amino acid sequencing. Cross-linking experiments allow to identify components of the postulated presenilin-APP-γ-secretase complex. By comparing protein profiles from isolated vesicles generated from material from the double deficient cell line, with that from wild type PS expressing ES cells, it is possible to identify proteins that are differentially present in the vesicles. Analyses by two-dimensional PAGE and amino acid sequencing of differentially detected protein spots will yield new proteins whose processing and transport is regulated by presenilins. Furthermore it is envisaged that proteins like γ-secretase or other proteases that are dependend on the presence of presenilins will be either decreased or increased in these samples. Further amino acid sequencing of these spots will yield γ-secretase and other protease candidates involved in RIP (regulated intramembrane proteolysis) (Brown *et al.*, 2000, Cell 100, 391).

7. Inhibitors of β-amyloid42 peptide production

In another example the mutant ES cell line is transformed with the wild type PS1 gene resulting in transformant 1 while transformant 2 is the mutant ES cell line genetically transformed with a specific familial Alzheimer's disease causing mutation in PS1or PS2 or combination of mutations. It is expected from the state of the art that transformant 2 predominantly produces the amyloid-β-42 form whereas transformant 1 mostly produces the non-pathogenic amyloid-β-40 peptide. These two transformants can be used in a differential drug screening approach to identify compounds which inhibit preferentially the amyloid-β-42 formation and not the amyloid-β-40 peptide generation. In a first screening with transformant 2 compounds are identified which specifically inhibit the formation of the amyloid-β-42 peptide. Specific monoclonal antibodies exist which can differentiate between the amyloid-β-42- and amyloid-β-40

peptide. In a second step the compounds identified in this first screening are applied on transformant 1. Subsequently specific compounds are identified which do not have an effect on the amyloid-β-40 secretion. Furthermore the system can be optimised in a high-throughput way so that large collections of existing chemical compound libraries can be quickly and efficiently validated. The discovery of such desirable compounds has traditionally been carried out either by random screening of molecules (produced through chemical synthesis or isolated from natural sources, for example, see K. Nakanishi, Acta Pharm. Nord, 1992, 4, 319), or by using a so-called "rational" approach involving identification of a lead-structure and optimization of its properties through numerous cycles of structural redesign and biological testing (for example see Testa, B. & Kier, L. B. Med. Res. Rev. 1991, 11, 35-48 and Rotstein, S. H. & Murcko, M. J. Med.Chem. 1993, 36, 1700). Since most useful drugs have been discovered not through the "rational" approach but through the screening or randomly chosen compounds, a hybrid approach to drug discovery has recently emerged which is based on the use of combinatorial chemistry to construct huge libraries of randomly-built chemical structures which are screened for specific biological activities. (Brenner, S & Lerner, R. A. Proc. Natl. Acad. Sci. USA 1992, 89, 5381).

II.Materials and Methods

In figure 4A the construct and the principle of the assay is schematically represented. DNA constructs (fig 4B) were generated as follows:

The fragment of the murine HES-1 promotor containing nucleotides 7 to 251 (J. Biol.Chem. 269,7, 5150-5156, 1994) was isolated by polymerase chain reaction (PCR) using the following oligonucleotides: 5'-CTCAGGCGCGCGCCATTG-3' and 5'-AGAGGTAGACAGGGGATTCC -3'. As template the pGL2HES1luc (Nature, 377,355-358, 1995) was used.

The ampified fragment was subcloned in a pGEM-T vector (Promega) and verified by sequencing. The coding sequence of the luciferase gene from the pGL2 basic vector (Promega) was subcloned (as a HindIII (blunded)- SalI fragment) downstream the HES-1 promotor (SpeI (blunded)-SalI vector) giving rise to pHES-1luc construct. The PGKneo selection marker cassette from Adra et al (Gene, 105, 263-267, 1991) was introduced in the SalI site of pHES-luc as a SalI-XhoI fragment resulting in pHES-1luc-neo to generate stable cell lines.

APP-NIC (fig 4A and 4B) fusion constructs, also called chimeric proteins, were made using the human APP695 sequence and the mouse Notch-1 sequence. The APP encoding sequence (residues -20bp to +1992bp, the ATG of the APP open reading frame being number 1) was constructed by joining the SmaI–SacI fragment encoding bases (-20bp to +1692) to a PCR fragment encoding bases (+1693bp to +1992bp) generating a unique EcorV site and a Myc tag (EQKLISEEDL) at the 3' end. Oligonucleotides used were: 5'-AACCACCGTGGAGCTCCTTC-3' and 5'-CCAAGCTTCTACAAGTCCTCTTCAGAAATCAGCTTTTGCTCGTTAACGATATCGTC AACCTCCACCACACCATG-3'. Three different cDNA fragments encoding part of the Notch-1 intracellular domain (+5286bp to +6291bp, +5286bp to 7251bp, 5286bp to 7554bp respectively, bp 1 being the ATG) were generated by PCR on a mouse brain cDNA library and subcloned into the EcoRV site giving rise to APP:NIC2, APP:NIC, and APP:NIC1 (see fig4B). Oligonucletides used were: 5'-CACCCGGGTTCCCTGAGGGTTTCAAAGT-3', 5'-CCGCACGATATCGTGGTG-3', 5'-GCGTTAACATCTGCCTGACTGGGCTC-3' and 5'-CAGTTAACGGTGGTGGGCGGGCTGGAGAT-3'. A SV40 polyadenylation signal (isolated from pSG5, Stratagene) was added as indicated in the figure 4B. The mouse Pgk-1 promotor (–456bp to -18bp, Gene, 60, 65-74, 1987) or the SV40 early promotor from pSG5 (Stratagene) was cloned in the unique SmaI site of the fusion constructs.

The K595N;M596L (numbering as in APP 695, the M encoded by ATG is 1) Swedisch mutation was introduced into the pSG5APP:NIC plasmid by site directed mutagenesis (Stratagene) using the following primer 5'-GGAGATCTCTGAAGTGAATCTGGATGCAGAATTCCGAC –3'. This construct was called pSG5APPSw:NIC. PSG5βA4 :NIC was generated by replacing the APP ectodomain of pSG5APP:NIC with the APPC99 stub. APPC99 contains the carboxyterminal 99 amino acid residues of the APP sequence (thus starts with the β-cleavage site in APP). To obtain the correct cleavage by signal peptidase in the APPC99 stub an extra DA motif was added between the signal sequence and the β-cleavage site. A NIC construct containing only the intracellular part of the chimeric protein was generated by PCR with the following oligonucleotides 5'-AGGATCCATGGTGCTGCTGTCCCGCAAGCGCCGGCGGCAGCATGGCCAGCTCT GGTTCCCTGAGGGTTTCAAAGTGT-3' and 5'-GCGTTAACATCTGCCTGACTGGGCTC-3. The fusion APP:NIC and the NIC construct were cloned in pCDNAzeo (invitrogen) to generate stable cell lines.

The complete cDNA maps of the pHes1-Luc construct and the promotorless APP:NICD are included.

The constructs discussed above were transfected in COS cells, in Hela cells, in ES cells, and in HEK293 cells. We discuss the Hela cells here in more detail as one example.

Hela (provided from the ATCC culture collection) cells were cultured in 12 well plates in DME/F12 medium (Gibco, BRL) supplementented with 10% Fetal Bovine Serum. Plasmids were transfected using Fugene according to the manufacturer (Roche). The transfection reagent: DNA ratio was 6: 1. Five hours before transfection the culture medium was replaced by DME/F12 without serum. Each well was transfected with a total of 300 ng DNA consisting of 50 ng pHes1-luc and 250 ng of one of the mNoth plasmids discussed above or empty pSG5 vector (control). Luciferase activity reflecting activation of the Hes-1 promotor fragment was measured 48 h after transfection with the luciferase assay system of Promega using a lumino meter. All experiments were performed in duplicate or triplicate and repeated at least two times. Luciferase induction factors were determined as the ratio between the mean luciferace activitities of the mNotch variants (as indicated) and the mean luciferace activities of the empty pSG5 vector.

Stable transfected HELA cells were obtained by electroporation (500V/cm, 960µF) of 3.10^6 cells in de precence of 10 µg linearized (SalI) pHes1luc-neo DNA. After 24 h 500 µg/ml G418 was supplemented to the media. Resistant cells were picked, expanded, frozen and analysed. Cell line 105 giving low background luciferase activity and a 10 fold induction factor (after transfection with pSG5APP :NIC) was reelectropored in the presence of 10 µg linearized (ScaI) pCDNAzeoAPP :NIC or pCDNAzeoNIC DNA. After positive selection in media containing zeocin (20 µg/ml) resistant cells were picked, expanded, froozen and analysed. Both APP :NIC cell lines 13 and 17 are at least stable for 8 passages and are resulting in a 50 fold induction factor. One NIC cell line 3 is stable for at least 4 passages, giving a 25-fold induction of luciferase activity.

PCT BDS/PSKO/052

Original (for SUBMISSION) - printed on 21.02.2001 11:49:32 AM

0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.91 (updated 01.01.2001)
0-2	International Application No.	
0-3	Applicant's or agent's file reference	BDS/PSKO/052

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	7
1-2	line	3
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	Vakgroep voor Moleculaire Biologie - Plasmidencollectie (BCCM/LMBP)
1-3-2	Address of depositary institution	Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium
1-3-3	Date of deposit	24 February 2000 (24.02.2000)
1-3-4	Accession Number	LMBP 5472CB
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	YES
0-4-1	Authorized officer	G. KOESTEL 15 MAR 2001

FOR INTERNATIONAL BUREAU USE ONLY

0-5	This form was received by the International Bureau on:	
0-5-1	Authorized officer	

Claims

1. A mutant embryonic stem cell line in which the γ-secretase activity is reduced by more than 90% compared to the γ-secretase activity in wild-type embryonic stem cell lines.

2. A mutant embryonic stem cell line according to claim 1, wherein said mutant embryonic stem cell line is a double mutant embryonic stem cell line derived from a presenilin1$^{-/-}$- presenilin 2$^{-/-}$ knock-out mouse.

3. A mutant embryonic stem cell line according to claim 1 or 2, wherein said γ-secretase activity is reduced by more than 99%.

4. A mutant embryonic stem cell line according to claim 1 or 2, wherein said γ-secretase activity is reduced by more than 99.9%.

5. A mutant embryonic stem cell line according to any of claims 1-4, wherein said stem cell line is differentiated.

6. A mutant embryonic stem cell line according to claims 1-5, wherein said stem cell line is differentiated into a post-mitotic neuron.

7. A mutant embryonic stem cell line according to claims 1-5, wherein said stem cell line is differentiated into an adipocyte.

8. A method to produce an embryonic stem cell line according to any of claims 1-7 comprising:

- rescuing blastocysts from double presenilin knockout mice, and
- cultivation of said blastocysts *in vitro*.

9. Use of an embryonic stem cell line according to any of claims 1-7 for identifying a gene coding for a protein having γ-secretase activity characterized in that:

- said embryonic stem cell line is transfected with at least one gene coding for a protein whose ability to have γ-secretase activity is sought to be determined, and

- said γ-secretase activity in said embryonic stem cell line is monitored.

10. Use of an embryonic stem cell line according to any of claims 1-7 for identifying a compound which specifically interferes with the formation of the Aβ42-peptide and not with the formation of the Aβ-40 peptide characterized in that:

- said embryonic stem cell line is transfected with at least one mutated gene coding for presenilin 1 and/or presenilin 2 and/or amyloid β precursor protein, and
- said transfected stem cell line is exposed to at least one compound whose ability to interfere with the formation of the Aβ-42 peptide and not with the formation of the Aβ-40 peptide is sought to be determined, and
- said formation of Aβ-42 peptide is monitored.

11. A method for the production of a pharmaceutical composition comprising the usage of an embryonic stem cell line according to claim 9 or 10 and further more mixing the gene or compound identified or a derivative or homologue thereof with a pharmaceutically acceptable carrier.

12. A transgenic mouse obtained by using an embryonic stem cell line according to any of claim 1 to 4 comprising:

- transfecting said mutant ES cell line with a pathogenic presenillin Alzheimer disease causing gene and,
- injecting the resulting transfected mutant ES cell line into a blastocyst and,
- implanting said injected blastocyst into a female mouse.

13. A reporter system to detect intramembrane proteolytic processing comprising:

- a chimeric molecule comprising a fusion between a transcription factor and a transmembrane domain that is known to be a substrate for proteolytic processing, and
- a reporter construct that detects said proteolytic processing of said chimeric transcription factor.

14. A reporter system according to claim 13 wherein said chimeric molecule comprises a fusion between the intracellular domain of Notch and the transmembrane domain of APP.

15. A reporter system according to claim 13 wherein said chimeric molecule is set forth by SEQ ID NO: 13.

16. Use according to claims 13-15 to screen for modulators and/or proteases for intramembrane proteolytic processing comprising:

- constructing a reporter cell line by transfecting a cell line with a reporter system according to claims 13-15 and,
- treating said reporter cell line with at least one compound or transfecting said reporter cell line with at least one gene and,
- comparing the expression of the reporter gene present in said reporter cell line with the expression of the reporter gene in the non-treated or non-transfected reporter cell line.

17. A method for the production of a pharmaceutical composition comprising the usage of a reporter system and a cell line according to claims 13-16 and further more mixing the modulator and/or protease identified or a derivative or homologue thereof with a pharmaceutically acceptable carrier.

18. A process to construct a reporter system according to claims 13-15 comprising:

- the construction of a chimeric transcription factor by splicing a genetic element encoding a transcription factor to a genetic element encoding a transmembrane domain that is known to be a substrate for proteolytic processing and,
- the construction of a reporter construct by splicing a genetic element that is responsive to said transcription factor to a reporter gene

Figure 1

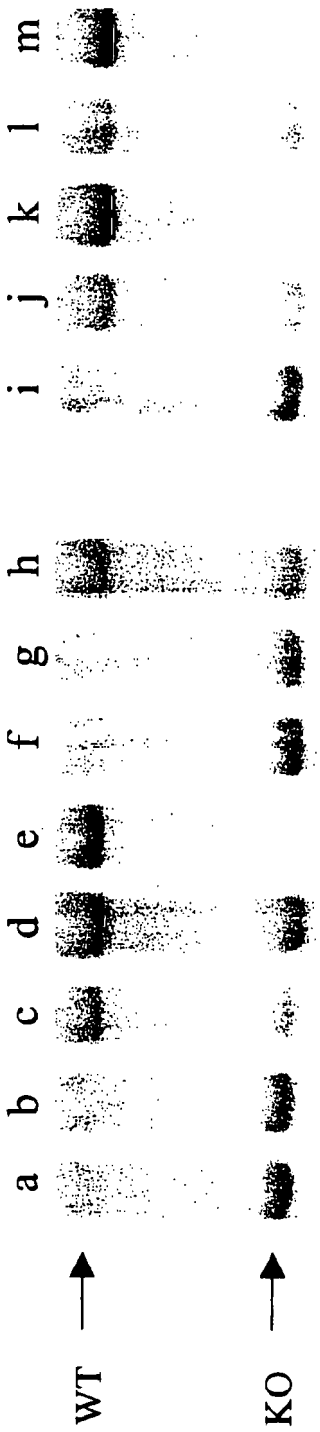


Figure 2

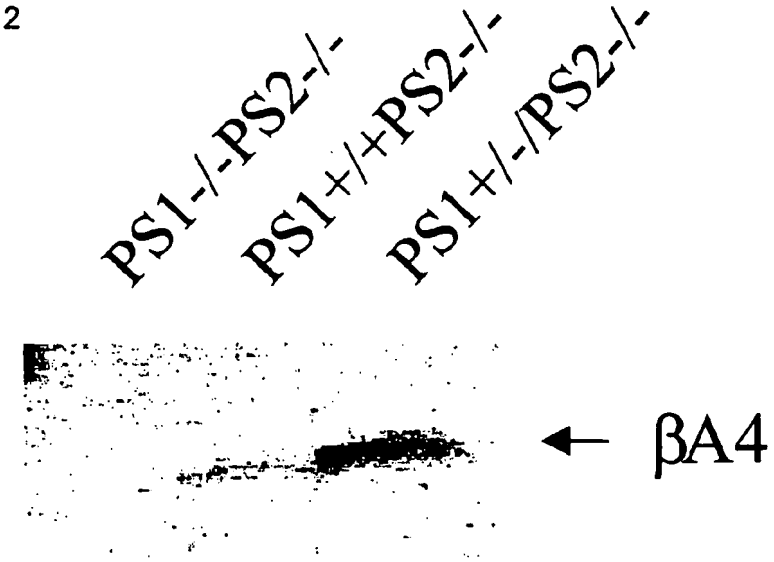


Figure 3

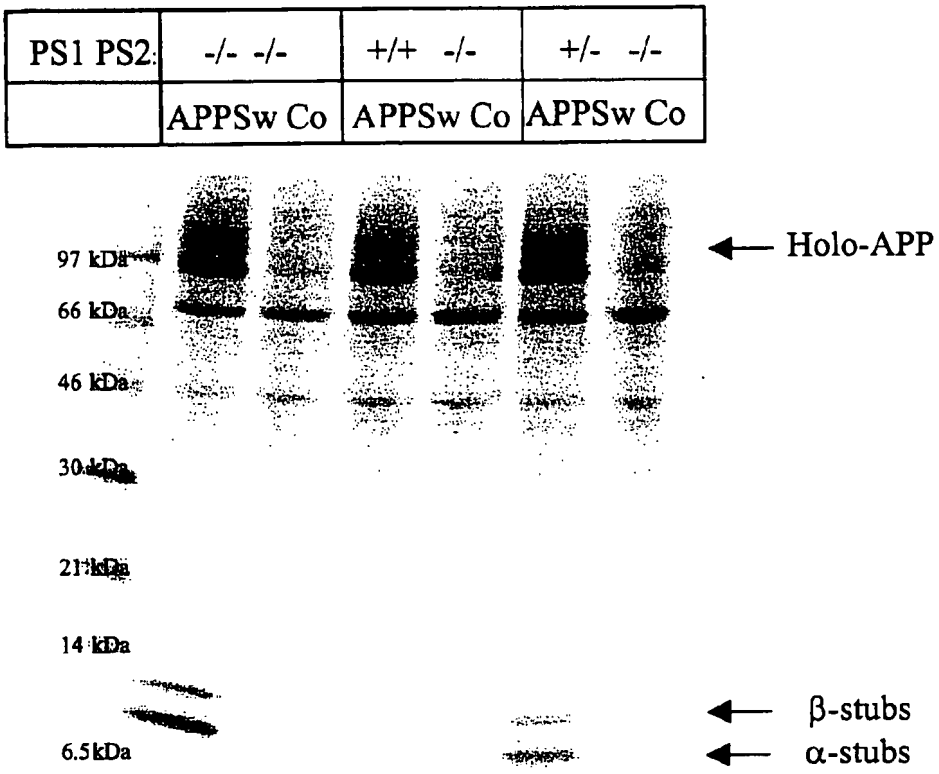


Figure 4 A

Principle

Chimeric APP : NIC construct

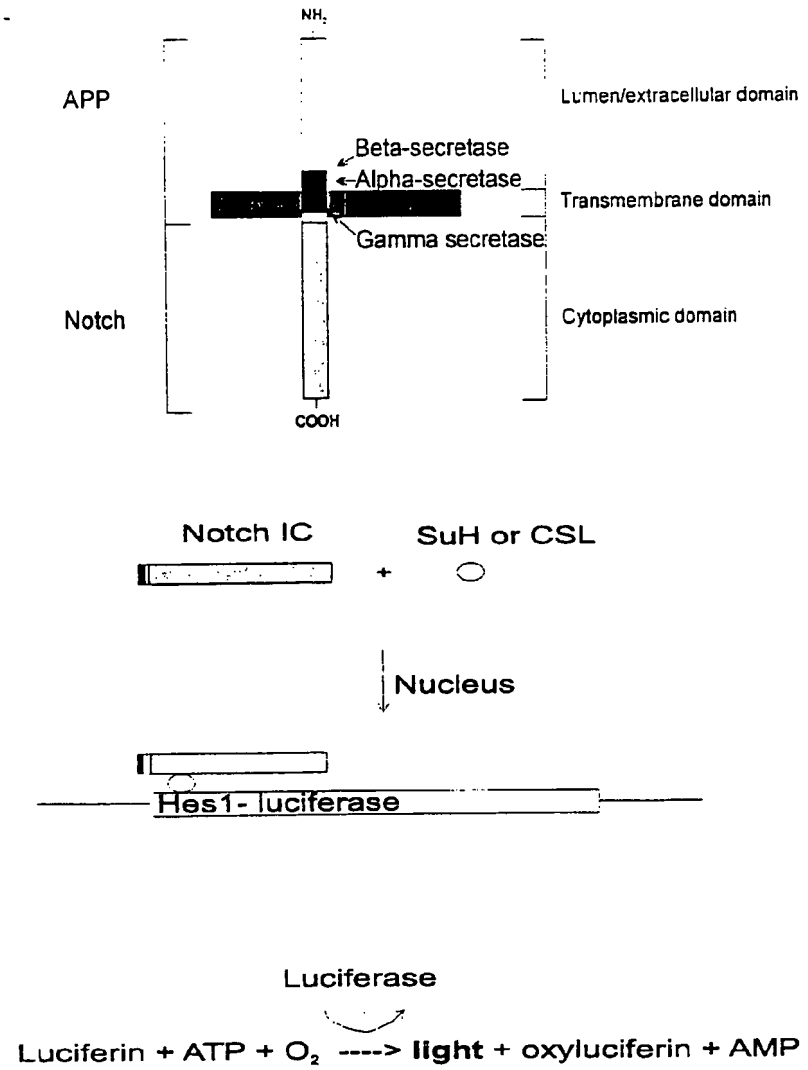


Figure 4 B

Reporterconstructs

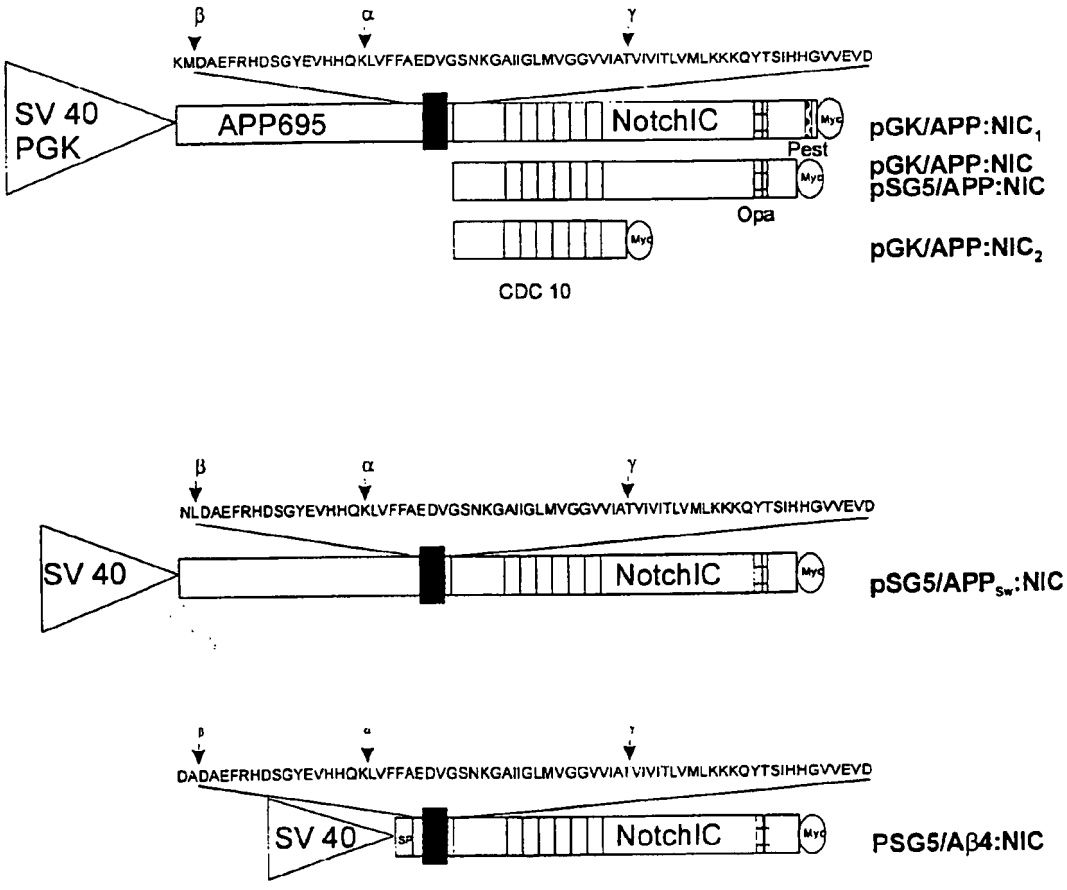


Figure 4 C

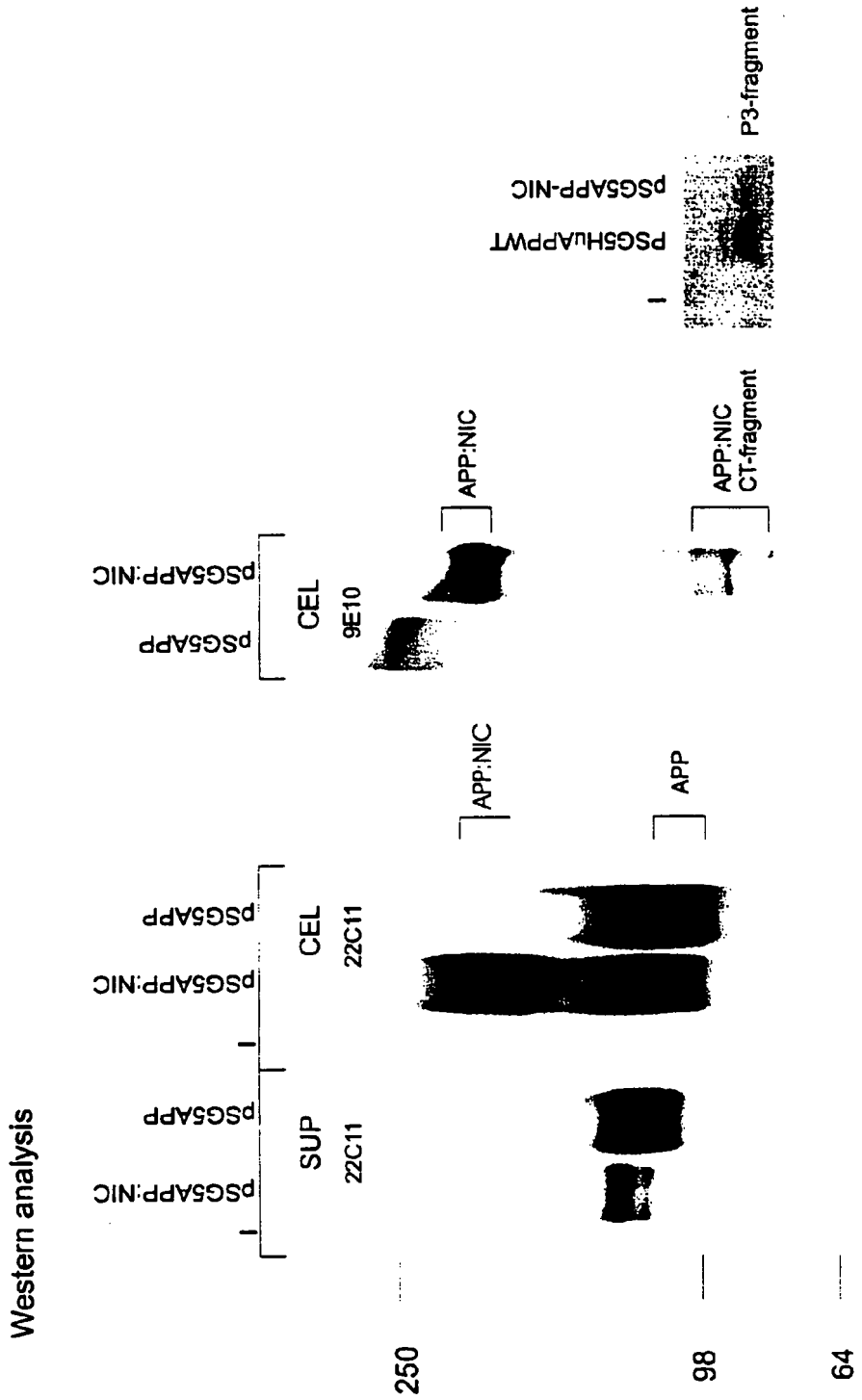
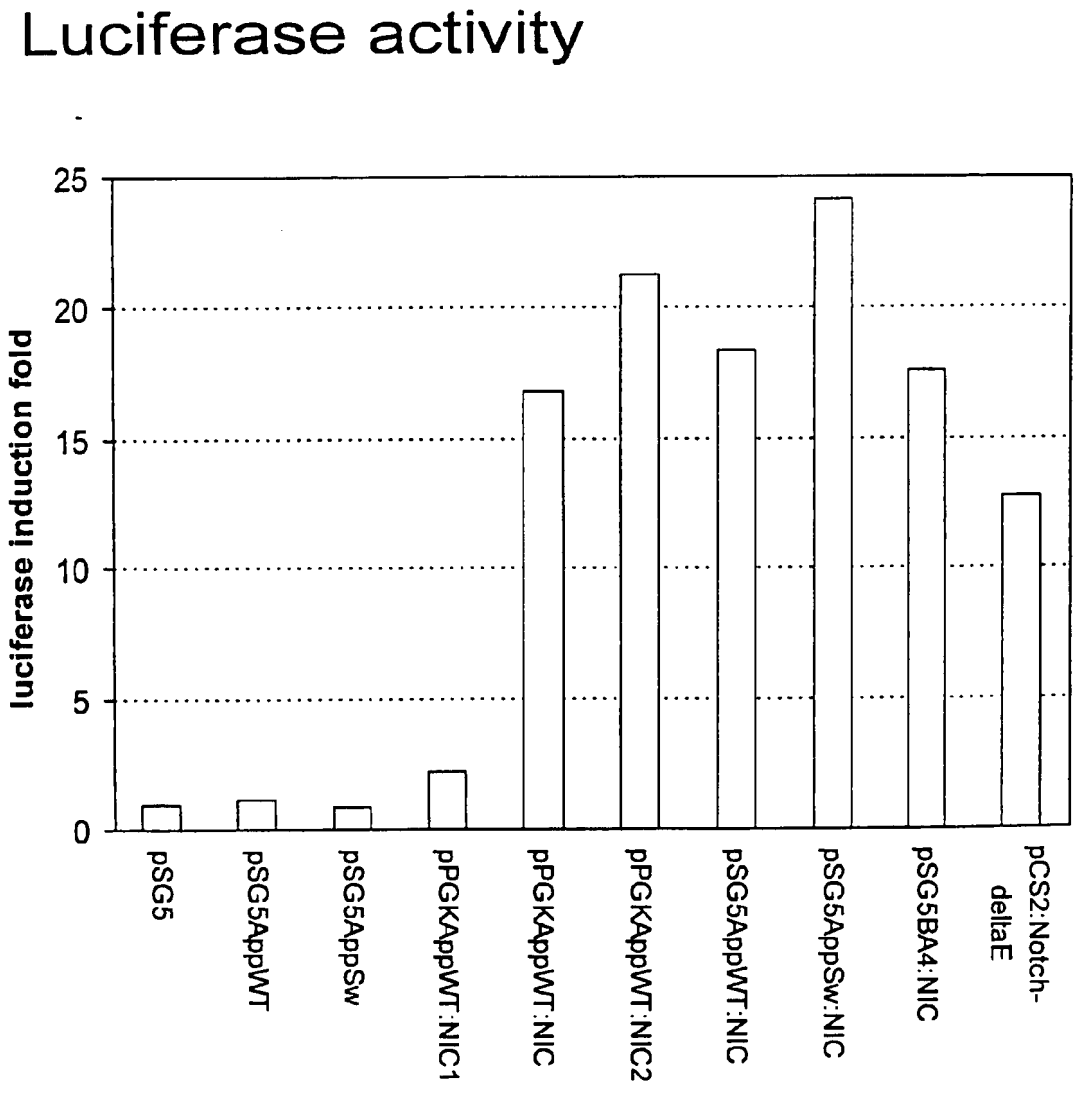

Western analysis
SUP
CEL
22C11
pSG5APP
pSG5APP-NIC
APP
APP:NIC
9E10
APP:NIC CT-fragment
P3-fragment
pSG5HuAPPwt
250
98
64

Figure 4 D


Luciferase activity
luciferase induction fold
25
20
15
10
5
0
pSG5
pSG5AppWT
pSG5AppSw
pPGKAppWT:NIC1
pPGKAppWT:NIC
pPGKAppWT:NIC2
pSG5AppWT:NIC
pSG5AppSw:NIC
pSG5BA4:NIC
pCS2:Notch-deltaE

SEQUENCE LISTING

<110> Vlaams Interuniversitair Instituut voor Biotechnol

<120> Presenilin deficient multipotent cell lines and screening methods for intramembrane regulated proteolytic activities using these lines

<130> BDS/PSKO/052

<140>
<141>

<150> 00200671.6
<151> 2000-02-29

<160> 13

<170> PatentIn Ver. 2.1

<210> 1
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: oligo PCR murine HES-1 promotor

<400> 1
ctcaggcgcg cgccattg 18

<210> 2
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: oligo 2 PCR murine HES-1 promotor

<400> 2
agaggtagac aggggattcc 20

<210> 3
<211> 10

<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: myc tag

<400> 3
Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu
1 5 10

<210> 4
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: oligo 1 PCR
APP

<400> 4
aaccaccgtg gagctccttc 20

<210> 5
<211> 73
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: oligo 2 PCR
APP

<400> 5
ccaagcttct acaagtcctc ttcagaaatc agcttttgct cgttaacgat atcgtcaacc 60
tccaccacac cat 73

<210> 6
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: oligo 1 PCR
Notch-1

<400> 6

cacccgggtt ccctgagggt ttcaaagt 28

<210> 7
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: oligo 2 PCR Notch-1

<400> 7
ccgcacgata tcgtggtg 18

<210> 8
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: oligo 3 PCR Notch-1

<400> 8
gcgttaacat ctgcctgact gggctc 26

<210> 9
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: oligo 4 PCR Notch-1

<400> 9
cagttaacgg tggtgggcgg gctggagat 29

<210> 10
<211> 38
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: oligo for mutagenesis

<400> 10
ggagatctct gaagtgaatc tggatgcaga attccgac 38

<210> 11
<211> 77
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: oligo 1 PCR Nic construct

<400> 11
aggatccatg gtgctgctgt cccgcaagcg ccggcggcag catggccagc tctggttccc 60
tgagggtttc aaagtgt 77

<210> 12
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: oligo 2 PCR Nic construc

<400> 12
ccgttaacat ctgcctgact gggctc 26

<210> 13
<211> 6868
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Fusion between intracellular domain of murine Notch and ecto- and transmembrane domains of human APP

<220>
<221> misc_feature
<222> (275)..(2266)
<223> Human APP sequence

<220>
<221> misc_feature
<222> (2147)..(2208)
<223> Transmembrane domain of APP

<220>
<221> misc_feature
<222> (2273)..(4249)
<223> Intracellular domain of murine Notch

<220>
<221> misc_feature
<222> (4256)..(4285)
<223> myc tag

<220>
<221> misc_signal
<222> (4286)..(4288)
<223> stop codon

<220>
<221> polyA_signal
<222> (4298)..(4444)
<223> Polyadenylation signal

<400> 13
gcgcccaata cgcaaaccgc ctctccccgc gcgttggccg attcattaat gcagctggca 60
cgacaggttt cccgactgga aagcgggcag tgagcgcaac gcaattaatg tgagttagct 120
cactcattag gcaccccagg ctttacactt tatgcttccg gctcgtatgt tgtgtggaat 180
tgtgagcgga taacaatttc acacaggaaa cagctatgac catgattacg aattcgagct 240
cggtacccgg ggatcaattc ccgccagggt ggcgatgctg cccggtttgg cactgctcct 300
gctggccgcc tggacggctc gggcgctgga ggtacccact gatggtaatg ctggcctgct 360
ggctgaaccc cagattgcca tgttctgtgg cagactgaac atgcacatga atgtccagaa 420
tgggaagtgg gattcagatc catcagggac caaaacctgc attgatacca aggaaggcat 480
cctgcagtat tgccaagaag tctaccctga actgcagatc accaatgtgg tagaagccaa 540
ccaaccagtg accatccaga actggtgcaa gcggggccgc aagcagtgca agacccatcc 600
ccactttgtg attccctacc gctgcttagt tggtgagttt gtaagtgatg cccttctcgt 660
tcctgacaag tgcaaattct tacaccagga gaggatggat gtttgcgaaa ctcatcttca 720
ctggcacacc gtcgccaaag agacatgcag tgagaagagt accaacttgc atgactacgg 780
catgttgctg ccctgcggaa ttgacaagtt ccgaggggta gagtttgtgt gttgcccact 840
ggctgaagaa agtgacaatg tggattctgc tgatgcggag gaggatgact cggatgtctg 900
gtggggcgga gcagacacag actatgcaga tgggagtgaa gacaaagtag tagaagtagc 960
agaggaggaa gaagtggctg aggtggaaga agaagaagcc gatgatgacg aggacgatga 1020
ggatggtgat gaggtagagg aagaggctga cgaaccctac gaagaagcca cagagagaac 1080
caccagcatt gccaccacca ccaccaccac cacagagtct gtggaagagg tggttcgagt 1140
tcctacaaca gcagccagta cccctgatgc cgttgacaag tatctcgaga cacctgggga 1200
tgagaatgaa catgcccatt tccagaaagc caaagagagg cttgaggcca agcaccgaga 1260

gagaatgtcc caggtcatga gagaatggga agaggcagaa cgtcaagcaa agaacttgcc 1320
taaagctgat aagaaggcag ttatccagca tttccaggag aaagtggaat ctttggaaca 1380
ggaagcagcc aacgagagac agcagctggt ggagacacac atggccagag tcgaagccat 1440
gctcaatgac cgccgccgcc tcgccctgga gaactacatc accgctctgc agcgctgttcc 1500
tcctcggcct cgtcacgtgt tcaatatgct aaagaagtat gtccgcgcag aacagaagga 1560
cagacagcac accctaaagc atttcgagca tgtgcgcatg gtggatccca agaaagccgc 1620
tcagatccgg tcccaggtta tgacacacct ccgtgtgatt tatgagcgca tgaatcagtc 1680
tctctccctg ctctacaacg tgcctgcagt ggccgaggag attcaggatg aagttgatga 1740
gctgcttcag aaagagcaaa actattcaga tgacgtcttg gccaacatga ttagtgaacc 1800
aaggatcagt tacggaaacg atgctctcat gccatctttg accgaaacga aaaccaccgt 1860
ggagctcctt cccgtgaatg gagagttcag cctggacgat ctccagccgt ggcattcttt 1920
tggggctgac tctgtgccag ccaacacaga aaacgaagtt gagcctgttg atgcccgccc 1980
tgctgccgac cgaggactga ccactcgacc aggttctggg ttgacaaata tcaagacgga 2040
ggagatctct gaagtgaaga tggatgcaga attccgacat gactcaggat atgaagttca 2100
tcatcaaaaa ttggtgttct ttgcagaaga tgtgggttca aacaaaggtg caatcattgg 2160
actcatggtg ggcggtgttg tcatagcgac agtgatcgtc atcaccttgg tgatgctgaa 2220
gaagaaacag tacacatcca ttcatcatgg tgtggtggag gttgacgatg ggttccctga 2280
gggtttcaaa gtgtcagagg ccagcaagaa gaagcggaga gagcccctcg gcgaggactc 2340
agtcggcctc aagcccctga agaatgcctc agatggtgct ctgatggacg acaatcagaa 2400
cgagtgggga gacgaagacc tggagaccaa gaagttccgg tttgaggagc cagtagttct 2460
ccctgacctg agtgatcaga ctgaccacag gcagtggacc cagcagcacc tggacgctgc 2520
tgacctgcgc atgtctgcca tggccccaac accgcctcag ggggaggtgg atgctgactg 2580
catggatgtc aatgttcgag gaccagatgg cttcacaccc ctcatgattg cctcctgcag 2640
tggagggggc cttgagacag gcaacagtga agaagaagaa gatgcacctg ctgtcatctc 2700
tgacttcatc taccagggcg ccagcttgca caaccagaca gaccgcaccg gggagaccgc 2760
cttgcacttg gctgcccgat actctcgttc agatcgtcga aagcgccttg aggccagtgc 2820
agatgccaac atccaggaca acatgggccg tactccgtta catgcagcag tttctgcaga 2880
tgctcagggt gtcttccaga tcctgctccg gaacagggcc acagatctgg atgcccgaat 2940
gcatgatggc acaactccac tgatcctggc tgcgcgcctg gccgtggagg gcatgctgga 3000
ggacctcatc aactcacatg ctgacgtcaa tgccgtggat gacctaggca agtcggcttt 3060
gcattgggcg gccgcggtga acaatgtgga tgctgctgtt gtgctcctga agaacggagc 3120
caacaaggac atcgagaaca acaaggagga gacttccctg ttcctgtcga tccgccgtga 3180
gagctatgag actgccaaag tgttgctgga ccactttgcc aaccgggaca tcacggatca 3240
catggaccga ttgccgcggg acatcgcaca ggagcgtatg caccacgata tcgtgcggct 3300
tttggatgag tacaacctgg tgcggtcccc acagctgcat ggcactgccc tgggtggcac 3360
acccactctg tctcccacac tctgctcgcc aaatggctac cctggcaatc tcaagtctgc 3420
cacacagggc aagaaggccc gcaagccaag caccaaaggg ctggcttgtg gtagcaagga 3480
agctaaggac ctcaaggcac ggaggaagag ttcccaggat ggcaagggct ggctgttgga 3540
cagctcgtcg agcatgctgt cgcctgtgga ctccctcgag tcaccccatg gctacttgtc 3600
agatgtggcc tcgcaccccc tcctcccctc cccattccag cagtctccat ccatgcctct 3660
cagccacctg cctggtatgc ctgacaccca cctgggcatc agccacttga atgtggcagc 3720
caagcctgag atggcagcac tggctggagg tagccggttg gcctttgagc accccccgcc 3780
acgcctctcc cacctgcctg tagcctccag tgcctgcaca gtgctgagta ccaatggcac 3840
cggggctatg aatttcaccg tgggtgcacc ggcaagcttg aatggccagt gtgagtggct 3900
tccccggctc cagaatggca tggtgcccag ccagtacaac ccactacggc cgggtgtgac 3960
gccgggcaca ctgagcacac aggcagctgg gctccagcat agcatgatgg ggccactaca 4020
cagcagcctc tccaccaata ccttgtcccc gattatttac cagggcctgc ccaacacacg 4080
gctggcaaca cagcctcacc tggtgcagac ccagcaggtg cagccacaga acttaccgct 4140

```
ccagccacag aacctgcagc caccatcaca gccacacctc agtgtgagct cggcagccaa 4200
cgggcacctg gggcggagct tcttgagtgg ggagcccagt caggcagatg ttaacgagca 4260
aaagctgatt tctgaagagg acttgtagaa gcttctagat cttattaaag cagaacttgt 4320
ctattgcagc ttataatggt tacaaataaa gcaatagcat cacaaatttc acaaataaag 4380
catttttttc actgcattct agttgtggtt tgtccaaact catcaatgta tcttatcatg 4440
tctggtcgac ctgcaggcat gcaagcttgg cactggccgt cgttttacaa cgtcgtgact 4500
gggaaaaccc tggcgttacc caacttaatc gccttgcagc acatcccccc ttcgccagct 4560
ggcgtaatag cgaagaggcc cgcaccgatc gcccttccca acagttgcgc agcctgaatg 4620
gcgaatggcg cctgatgcgg tattttctcc ttacgcatct gtgcggtatt tcacaccgca 4680
tatggtgcac tctcagtaca atctgctctg atgccgcata gttaagccag ccccgacacc 4740
cgccaacacc cgctgacgcg ccctgacggg cttgtctgct cccggcatcc gcttacagac 4800
aagctgtgac cgtctccggg agctgcatgt gtcagaggtt ttcaccgtca tcaccgaaac 4860
gcgcgagacg aaagggcctc gtgatacgcc tatttttata ggttaatgtc atgataataa 4920
tggtttctta gacgtcaggt ggcacttttc ggggaaatgt gcgcggaacc cctatttgtt 4980
tatttttcta aatacattca aatatgtatc cgctcatgag acaataaccc tgataaatgc 5040
ttcaataata ttgaaaaagg aagagtatga gtattcaaca tttccgtgtc gcccttattc 5100
ccttttttgc ggcattttgc cttcctgttt ttgctcaccc agaaacgctg gtgaaagtaa 5160
aagatgctga agatcagttg ggtgcacgag tgggttacat cgaactggat ctcaacagcg 5220
gtaagatcct tgagagtttt cgccccgaag aacgttttcc aatgatgagc acttttaaag 5280
ttctgctatg tggcgcggta ttatcccgta ttgacgccgg gcaagagcaa ctcggtcgcc 5340
gcatacacta ttctcagaat gacttggttg agtactcacc agtcacagaa aagcatctta 5400
cggatggcat gacagtaaga gaattatgca gtgctgccat aaccatgagt gataacactg 5460
cggccaactt acttctgaca acgatcggag gaccgaagga gctaaccgct tttttgcaca 5520
acatgggggа tcatgtaact cgccttgatc gttgggaacc ggagctgaat gaagccatac 5580
caaacgacga gcgtgacacc acgatgcctg tagcaatggc aacaacgttg cgcaaactat 5640
taactggcga actacttact ctagcttccc ggcaacaatt aatagactgg atggaggcgg 5700
ataaagttgc aggaccactt ctgcgctcgg cccttccggc tggctggttt attgctgata 5760
aatctggagc cggtgagcgt gggtctcgcg gtatcattgc agcactgggg ccagatggta 5820
agccctcccg tatcgtagtt atctacacga cggggagtca ggcaactatg gatgaacgaa 5880
atagacagat cgctgagata ggtgcctcac tgattaagca ttggtaactg tcagaccaag 5940
tttactcata tatactttag attgatttaa aacttcattt ttaatttaaa aggatctagg 6000
tgaagatcct ttttgataat ctcatgacca aaatcccttа acgtgagttt tcgttccact 6060
gagcgtcaga ccccgtagaa aagatcaaag gatcttcttg agatcctttt tttctgcgcg 6120
taatctgctg cttgcaaaca aaaaaaccac cgctaccagc ggtggtttgt ttgccggatc 6180
aagagctacc aactcttttt ccgaaggtaa ctggcttcag cagagcgcag ataccaaata 6240
ctgtccttct agtgtagccg tagttaggcc accacttcaa gaactctgta gcaccgccta 6300
catacctcgc tctgctaatc ctgttaccag tggctgctgc cagtggcgat aagtcgtgtc 6360
ttaccgggtt ggactcaaga cgatagttac cggataaggc gcagcggtcg ggctgaacgg 6420
ggggttcgtg cacacagccc agcttggagc gaacgaccta caccgaactg agatacctac 6480
agcgtgagct atgagaaagc gccacgcttc ccgaagggag aaaggcggac aggtatccgg 6540
taagcggcag ggtcggaaca ggagagcgca cgagggagct tccagggggа aacgcctggt 6600
atctttatag tcctgtcggg tttcgccacc tctgacttga gcgtcgattt ttgtgatgct 6660
cgtcaggggg gcggagccta tggaaaaacg ccagcaacgc ggcctttttа cggttcctgg 6720
ccttttgctg gccttttgct cacatgttct ttcctgcgtt atcccctgat tctgtggata 6780
accgtattac cgcctttgag tgagctgata ccgctcgccg cagccgaacg accgagcgca 6840
gcgagtcagt gagcgaggaa gcggaaga                                    6868
```

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/02127

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 C12N15/10 A61K35/12 A01K67/027 C12N15/63
C07K14/47 C12N15/12 A61P25/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE STROOPER BART ET AL: "Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein." NATURE (LONDON), vol. 391, no. 6665, 22 January 1998 (1998-01-22), pages 387-390, XP001010557 ISSN: 0028-0836 cited in the application page 387, right-hand column, line 1 -page 388, left-hand column, line 3 page 388, right-hand column, line 21 -page 390, left-hand column, line 5 --- -/--	1,2,8,12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 July 2001	09/08/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/02127

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HERREMAN AN ET AL: "Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 96, no. 21, 12 October 1999 (1999-10-12), pages 11872-11877, XP002173228 Oct. 12, 1999 ISSN: 0027-8424 cited in the application abstract page 11875, right-hand column, last paragraph -page 11877, left-hand column, paragraph 1 ---	1,2,8,12
A	DE STROOPER BART ET AL: "A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain." NATURE (LONDON), vol. 398, no. 6727, 8 April 1999 (1999-04-08), pages 518-522, XP001010555 ISSN: 0028-0836 the whole document ---	1,10,13
A	KIMBERLY W TAYLOR ET AL: "The transmembrane aspartates in presenilin 1 and 2 are obligatory for gamma-secretase activity and amyloid beta-protein generation." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 275, no. 5, 4 February 2000 (2000-02-04), pages 3173-3178, XP002173229 ISSN: 0021-9258 the whole document ---	1-4,10
A	WOLFE MICHAEL S ET AL: "Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity." NATURE (LONDON), vol. 398, no. 6727, 8 April 1999 (1999-04-08), pages 513-517, XP001010554 ISSN: 0028-0836 the whole document --- -/—	1-4

1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/02127

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WOLFE MICHAEL S ET AL: "Peptidomimetic probes and molecular modeling suggest that Alzheimer's gamma-secretase is an intramembrane-cleaving aspartyl protease." BIOCHEMISTRY, vol. 38, no. 15, 13 April 1999 (1999-04-13), pages 4720-4727, XP002173230 ISSN: 0006-2960 the whole document	1-4
A	LICHTENHALER S F ET AL: "NOVEL SUBSTRATE FOR ANALYZING ALZHEIMER'S DISEASE GAMMA-SECRETASE" FEBS LETTERS,ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM,NL, vol. 453, no. 3, 1999, pages 288-292, XP000937693 ISSN: 0014-5793 abstract page 288, left-hand column, paragraph 1 -page 289, right-hand column, paragraph 1 page 291, left-hand column, paragraph 2 -page 292, left-hand column, last paragraph	12,13,16
A	WO 98 51781 A (PLOEG LEONARDUS H T V D ;SISODIA SANGRAM S (US); QIAN SU (US); WON) 19 November 1998 (1998-11-19) page 3, line 26 -page 7, line 6; examples 1-6,13,14	1,2,12
A	DANI C ET AL: "Differentiation of embryonic stem cells into adipocytes in vitro." JOURNAL OF CELL SCIENCE, vol. 110, no. 11, 1997, pages 1279-1285, XP002173231 ISSN: 0021-9533 the whole document	5,7,8
A	OKABE S ET AL: "DEVELOPMENT OF NEURONAL PRECURSOR CELLS AND FUNCTIONAL POSTMITOTIC NEURONS FROM EMBRYONIC STEM CELLS IN VITRO" MECHANISMS OF DEVELOPMENT,IE,ELSEVIER SCIENCE IRELAND LTD, vol. 59, no. 1, 1996, pages 89-102, XP000615956 ISSN: 0925-4773 cited in the application the whole document	5,6,8

-/--

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/02127

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 00 34511 A (AVENTIS PHARMA GMBH) 15 June 2000 (2000-06-15) abstract page 3, line 4 -page 6, line 6 examples 1-3	1,3,4, 9-11,13
P,X	WO 00 68416 A (BOEHRINGER INGELHEIM PHARMA ;HAASS CHRISTIAN (DE); PESOLD BRIGITTE) 16 November 2000 (2000-11-16) abstract page 3, line 20-35 page 6, line 28 -page 8, line 28; figures 4,5; examples 1,2	13
P,X	HERREMAN AN ET AL: "Total inactivation of gamma-secretase activity in presenilin-deficient embryonic stem cells." NATURE CELL BIOLOGY, vol. 2, no. 7, July 2000 (2000-07), pages 461-462, XP001015216 ISSN: 1465-7392 the whole document	1-18
P,X	ZHANG ZHUOHUA ET AL: "Presenilins are required for gamma-secretase cleavage of beta-APP and transmembrane cleavage of Notch-1." NATURE CELL BIOLOGY, vol. 2, no. 7, July 2000 (2000-07), pages 463-465, XP001015214 ISSN: 1465-7392 the whole document	1,10,12
P,X	ZHANG J ET AL: "APP chimeric proteins containing the Notch trans-membrane domain are cleaved by gamma-secretase." SOCIETY FOR NEUROSCIENCE ABSTRACTS, vol. 26, no. 1-2, 2000, pages Abstract No.-180.12, XP001014404 30th Annual Meeting of the Society of Neuroscience;New Orleans, LA, USA; November 04-09, 2000 ISSN: 0190-5295 abstract	13-18

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir ational Application No
PCT/EP 01/02127

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9851781 A	19-11-1998	EP 0981602 A	01-03-2000
WO 0034511 A	15-06-2000	DE 19856261 C	30-03-2000
		AU 1968000 A	26-06-2000
WO 0068416 A	16-11-2000	DE 19920514 A	16-11-2000